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Stimulating photosynthetic processes increases productivity and water use efficiency in the field

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Short title: Improving photosynthesis and yield

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One sentence summary: Simultaneous stimulation of RuBP regeneration and electron transport results in improvements in biomass yield in glasshouse and field grown tobacco.

Abstract

Previous studies have demonstrated that independent stimulation of either electron transport or RuBP regeneration can increase the rate of photosynthetic carbon assimilation and plant biomass. In this paper, we present evidence that a multi-gene approach to simultaneously manipulate these two processes provides a further stimulation of photosynthesis. We report on the introduction of the cyanobacterial bifunctional enzyme fructose-1, 6-bisphosphatase/sedoheptulose-1,7-bisphosphatase or overexpression of the plant enzyme sedoheptulose-1,7-bisphosphatase, together with expression of the red algal protein cytochrome $c_{6,}$ and show that a further increase in biomass accumulation under both glasshouse and field conditions can be achieved. Furthermore, we provide evidence that stimulation of both electron transport and RuBP regeneration can lead to enhanced intrinsic water use efficiency under field conditions.

Keywords: SBPase; FBP/SBPase; Calvin-Benson cycle; cytochrome c_6; chlorophyll fluorescence imaging; transgenic; electron transport; biomass; water use efficiency.

Yield potential of seed crops grown under optimal management practices, and in the absence of biotic and abiotic stress, is determined by incident solar radiation over the growing season, the efficiency of light interception, energy conversion efficiency and partitioning or harvest index. For the major crops, the only component not close to the theoretical maximum is energy conversion efficiency, which is determined by gross canopy photosynthesis minus respiration. This highlights photosynthesis as a target for improvement to raise yield potential in major seed crops[1-3].

Transgenic experiments and modelling studies have provided compelling evidence that increasing the levels of photosynthetic enzymes in the Calvin Benson (CB) cycle has the potential to impact photosynthetic rate and yield[1,2,4-15]. Over-expression of SBPase in tobacco[5,7,8], Arabidopsis[9], tomato[15] and wheat[16] has demonstrated the potential of manipulating the expression of CB cycle enzymes and specifically the regeneration of RuBP to increase growth, biomass (30-42%) and even seed yield (10-53%). Similarly, overexpression of other enzymes including FBPA[14], cyanobacterial SBPase, FBPase[17] and the bifunctional fructose-1,6-bisphosphatases/sedoheptulose-1,7-bisphosphatase (FBP/SBPase[4,18,19]) in a range of species has shown that increasing photosynthesis increases yield. In addition to manipulation of CB cycle genes, increasing photosynthetic electron transport has also been shown to have a beneficial effect on plant growth. Overexpression of the Rieske FeS protein -a key component of the cytochrome b_6f complex- in Arabidopsis, has previously been shown to lead to increases in electron transport rates, CO_2 assimilation, biomass and seed yield[20]. Similar results were also observed when the Rieske FeS protein was over-expressed in the C4 plant *Setaria viridis* demonstrating that this manipulation has the potential to have a positive effect in both C3 and C4 species [21]. Furthermore, the introduction of the algal cytochrome c_6 protein into Arabidopsis and tobacco resulted in

63 increased growth[22,23]. In cytochrome c_6 expressing transgenic plants, the electron transport
64 rate was increased along with ATP, NADPH, chlorophyll, starch content, and capacity for
65 CO_2 assimilation. Higher plants have been proposed to have lost the cytochrome c_6 protein
66 through evolution, but in green algae and cyanobacteria, which have genes for both
67 cytochrome c_6 and plastocyanin (PC), cytochrome c_6 has been shown to replace PC as the
68 electron transporter connecting the cytochrome b_6f complex with PSI under Cu deficiency
69 conditions[24,25]. There is evidence showing that PC can limit electron transfer between
70 cytochrome b_6f complex and PSI[26], and in Arabidopsis, it has been shown that introduced
71 algal cytochrome c_6 is a more efficient electron donor to P700 than PC[22]. This evidence
72 suggests the introduction of the cytochrome c_6 protein in higher plants as a viable strategy for
73 improving photosynthesis.

74 This paper aims to test the hypothesis that combining an increase in the activity of a
75 CB cycle enzyme, specifically enhancing RuBP regeneration, together with stimulation of the
76 electron transport chain can boost photosynthesis and yield above that observed when these
77 processes are targeted individually. *Nicotiana tabacum* plants expressing the cyanobacterial
78 FBP/SBPase or the higher plant SBPase, and the algal cytochrome c_6 were generated using
79 two different tobacco cultivars. The analysis presented here demonstrates that the
80 simultaneous stimulation of electron transport and RuBP regeneration leads to a significant
81 increase in photosynthetic carbon assimilation, and results in increased biomass and yield
82 under both glasshouse and field conditions.
83

Production and Selection of Tobacco Transformants

Previous differences observed in the biomass accumulation between Arabidopsis and tobacco overexpressing SBPase and SBPase plus FBPA[8,9] led us to explore the effect of similar manipulations (RuBP regeneration by overexpression of SBPase or introduction of the cyanobacterial FBP/SBPase, together with enhanced electron transport) in two different tobacco cultivars with different growth habits: *N. tabacum* cv. Petite Havana, with indeterminate growth, and *N. tabacum* cv. Samsun, with determinate growth. Sixty lines of cv. Petit Havana, and up to fourteen lines of cv. Samsun were generated per construct and T0 and T1 transgenic tobacco were screened by qPCR and immuno-blot analysis to select independent lines with expression of the transgenes (data not shown).

N. tabacum cv. Petit Havana T2/T3 progeny expressing FBP/SBPase (S_B; lines S_B03, S_B06, S_B21, S_B44) or cytochrome c_6 (C_6; lines C15, C41, C47, C50) and cv. Samsun lines expressing SBPase + cytochrome c_6 (SC_6, lines SC1, SC2 and SC3) were produced by agrobacterium transformation. *N. tabacum* cv. Petit Havana plants expressing both S_B and C_6 were generated by crossing S_B lines (S_B06, S_B21, S_B44) with C_6 lines (C15, C47, C50) to generate four independent S_BC_6 lines: S_BC1 (S_B06 x C47), S_BC2 (S_B06 x C50), S_BC3 (S_B44 x C47) and S_BC6 (S_B21 x C15). Semi-quantitative RT-PCR was used to detect the presence of the FBP/SBPase transcript in lines S_B and S_BC_6, cytochrome c_6 in lines C_6, S_BC_6 and SC_6, and SBPase in lines S and SC_6 (**Supplementary Fig. 1**). The selected S_B and S_BC_6 lines were shown to accumulate FBP/SBPase protein, and S and SC_6 to overexpress the SBPase protein by immunoblot analysis (**Fig. 1a and Supplementary Fig. 2**). In addition to immunoblot analysis, we analysed total extractable FBPase activity in the leaves of the cv. Petite Havana T2/T3 & F3 homozygous progeny lines used to determine chlorophyll fluorescence and photosynthetic parameters. This analysis showed that these plants (S_B and S_BC_6) had increased levels of FBPase activity ranging from 34 to 47% more than the control plants (**Fig.**

1c). The full set of assays showing the variation in FBPase activities between plants can be seen in supplemental data (**Supplementary Fig. 3**). The S and SC_6 lines were from the same generation of transgenic plants used in a previous study and shown to have increased SBPase activity[8]. The cytochrome c_6 antibody (raised against a peptide from the *Porphyra umbilicalis* protein) was unable to detect less than 60 ng of purified cytochrome c_6 protein extracted from *E. coli* (**Supplementary Fig. 4**), and immunoblotting of leaf extracts did not result in a signal. However, when semi-purified extracts from lines C15, C41 and C47 were used, a band of the expected molecular weight was identified in semi-purified extracts from lines C15, C41 and C47, providing qualitative confirmation of the presence of cytc6 in the transgenic tobacco plants (**Fig. 1b and Supplementary Fig. 5a**). No bands were observed in semi-purified extracts from control (CN) plants. To provide further evidence of the presence of introduced cytochrome c_6 protein a spectral scan was run using the semi-purified protein extracts of C_6 and CN plants; the soret peak at 420 nm demonstrated the presence of the heme group and was only detectable in the C_6 transgenic plants and not in the CN plants. (**Supplementary Fig. 5b**). Additionally, a physiological assay probing the response of photosynthesis during light induction was performed. CN and C_6 plants were provided with saturating light and [CO_2] following a period of darkness. The C_6 plants were shown to have both a more rapid response and greater rate of net CO_2 assimilation compared with CN plants (**Supplementary Fig. 6a & 6d).** The faster increase in *A* was accompanied by a quicker rise in the operating efficiency of both PSII (F_q'/F_m') and PSI (YI) providing evidence that in these plants electron flow through both photosystems was increased. This increase in electron transport could contribute to the higher *A* rates observed by providing the required energy (ATP) and reductant (NADPH). This response was further accelerated in S_BC_6 transgenic plants mostly likely due to the increased sink capacity provided by CB cycle activity (**Supplementary Fig. 6**).

Chlorophyll fluorescence analysis confirmed that in young plants, the operating efficiency of photosystem two (PSII) photochemistry F_q'/F_m' at an irradiance of 600-650 μmol m^{-2} s^{-1} was significantly higher in all selected lines compared to either WT or null segregant controls (**Fig. 1d, e**). However, the F_q'/F_m' values of the S_BC_6 and SC_6 lines, were not significantly different from the F_q'/F_m' values obtained from the plants expressing individually FBP/SBPase (S_B), cytochrome c_6 (C_6) or SBPase (S).

Stimulation of electron transport and RuBP regeneration increases photosynthesis

Transgenic lines selected based on the initial screens described above were grown in the glasshouse, in natural light supplemented to provide illumination between 400-1000 μmol m^{-2} s^{-1}. The rate of net CO_2 assimilation (A) and F_q'/F_m' was determined as a function of internal CO_2 concentration (C_i), in mature and developing leaves of *N. tabacum* cv. Samsun (S and SC_6) and in mature leaves of *N. tabacum* cv. Petit Havana (S_B, C_6 and S_BC_6) (**Fig. 2**). The transgenic plants displayed greater CO_2 assimilation rates than those of the control (CN) plants. A was 15% higher than the controls in the mature leaves of the SC_6, at a C_i of approximately 300 μmol mol^{-1} (the C_i prevailing at current atmospheric [CO_2]) (**Fig. 2b**). The developing leaves of the SC_6 plants also showed significant increases in PSII operating efficiency (F_q'/F_m') and in the PSII efficiency factor (F_q'/F_v'; which is determined by the ability of the photosynthetic apparatus to maintain Q_A in the oxidized state and therefore a measure of photochemical quenching) when compared to control plants (**Fig. 2c**). Interestingly, in mature leaves of the cv. Samsun transgenic plants, the differences in assimilation rates and in the operating efficiency of PSII photochemistry between the transgenic and the CN plants were smaller than in the developing leaves. Only the S transgenic plants displayed a higher average value for F_q'/F_m' and F_q'/F_v' than the CN plants

159 at all CO_2 concentrations measured. In contrast, the mature leaves of SC_6 plants displayed
160 F_q'/F_v' values higher than the control only at C_i levels between 300 and 900 μmol mol^{-1} (**Fig.**
161 **2b**).

162 Similar trends were shown for the *N. tabacum* cv. Petit Havana transgenic plants,
163 which displayed higher average values of A and F_q'/F_m' than the CN (**Fig. 2a**). In the leaves
164 of the S_BC_6 plants (cv. Petit Havana) these significant increases were similar to the
165 developing leaves of the SC_6 lines (cv. Samsun). No significant differences in PSII maximum
166 efficiency (F_v'/F_m') were observed between the CN and the transgenics in either cultivar.

167 The developing leaves of both the S and SC_6 plants (cv. Samsun) showed a
168 significant increase in both the maximum electron transport and RuBP regeneration rate
169 (J_{max}) and maximum assimilation (A_{max}) when compared the control plants (**Table 1**). The
170 mature leaves of the SC_6 (cv. Samsun) and S_BC_6 (cv. Petite Havana) transgenics also
171 displayed a significantly higher A_{max} than the CN, and higher average values for $Vc_{max,}$ and
172 J_{max} were also evident in these leaves. These results showed that simultaneous stimulation of
173 electron transport and RuBP regeneration by expression of cytochrome c_6 in combination
174 with FBP/SBPase or SBPase has a greater impact on photosynthesis than the single
175 manipulations in all plants analysed.
176
177 **Stimulation of electron transport and RuBP regeneration improves growth**
178 In parallel experiments, plants expressing FBP/SBPase (S_B), cytochrome c_6 (C_6) and
179 both (S_BC_6) (*N. tabacum* cv. Petite Havana) and plants expressing SBPase (S) and SBPase +
180 cytochrome c_6 (SC_6) (*N. tabacum* cv. Samsun) were grown in the glasshouse for four and six
181 weeks respectively before harvesting. Height, leaf number, total leaf area and above ground
182 biomass were determined (**Fig 3** and **Supplementary Fig 7**). All of the transgenic plants
183 analysed here displayed increased height when compared to CN plants. Plants expressing

cytochrome c_6 (C_6, S_BC_6, (cv. Petite Havana) and SC_6 (cv. Samsun)) had a significant increase in leaf area and in stem and leaf biomass compared to their respective controls (**Fig.3** and **Supplementary Fig. 8,9**). In the S_B transgenic plants (cv. Petite Havana) only the biomass of the stem was greater than the CN plants. Notably the S_BC_6 and SC_6 transgenics displayed significantly greater leaf area than the single S_B and S transgenic plants respectively. The total increase in above ground biomass when compared to CN group was 35% in S_B, 44% in C_6 and 9% in S, with consistently higher means in the double manipulations S_BC_6 (52%) and SC_6 (32%) (**Fig.3**).

Expression of FBP/SBPase and cytochrome c_6 increases growth and water use efficiency

To test whether the increases in biomass observed in these transgenic plants under glasshouse conditions could be reproduced in a field environment, a subset of lines was selected for testing in the field. Since the larger percentage increases in biomass were displayed by the manipulations in *N. tabacum* cv. Petit Havana, these plants were selected and tested in three field experiments in two different years (2016 and 2017).

In 2016, a small-scale replicated control experiment was carried out to evaluate vegetative growth in the field, in the lines expressing single gene constructs for FBP/SBPase (S_B) and cytochrome c_6 (C_6) (**Supplementary Fig. 14a**). Plants were germinated and grown under controlled environment conditions for 25 d before being moved to the field. After 14 d in the field, plants were harvested at an early vegetative stage and plant height, total leaf area and above ground biomass were measured (**Fig. 4 (a-c)** and **Supplementary Fig. 10a**). These data revealed that the S_B and C_6 plants showed an increase in height, leaf area and above ground biomass of 27%, 35% and 25% respectively for S_B and 50%, 41% and 36% respectively for C_6 when compared to CN plants.

In 2017, two larger scale, randomized block design field experiments were carried out to evaluate performance in the S_B, C_6 and S_BC_6 plants compared to CN plants (**Supplementary Fig.14b**). Plants were grown from seed in the glasshouse for 33 d, and then moved to the field and allowed to grow until the onset of flowering (further 24-33 d), before harvesting. In **Fig. 4d-i** it can be seen that the S_B and C_6 plants harvested after the onset of flowering did not display any significant increases in height, leaf area or biomass when compared to CN plants. Interestingly, plants expressing both FBP/SBPase and cytochrome c_6 (S_BC_6), displayed a significant increase in a number of growth parameters; with 13%, 17% and 27% increases in height, leaf area and above ground biomass respectively when compared to CN plants.

Additionally, in the 2017 field experiments *A* as a function of C_i at saturating light (A/C_i) was determined. In the 2017 Experiment 1 a significant increase in *A* was observed in S_B and C_6 plants without differences in PSII operating efficiency (F_q'/F_m') (**Fig. 5a**). However, in the 2017 Experiment 2, no differences in *A* or in F_q'/F_m' values were evident in the C_6 and S_BC_6 plants when compared to the CN plants (**Fig. 5b**). Analysis of *A* as a function of light (*A/Q*) showed either small or no significant differences in *A* between genotypes (**Fig. 6a** and **Supplementary Fig 11a**). Interestingly, g_s in the S_BC_6 plants was significantly lower than for the C_6 and CN plants at light intensities above 1000 μmol m^{-2} s^{-1} (**Fig 6b**), resulting in a significant increase in intrinsic water use efficiency (*iWUE)* for S_BC_6 plants (**Fig 6d**). No significant differences in *iWUE* were observed for S_B or C_6 transgenic plants (**Fig 6d** and **Supplementary Fig 11d**).

DISCUSSION

In this study, we describe the generation and analysis of transgenic plants with simultaneous increases in electron transport and improved capacity for RuBP regeneration, in two different tobacco cultivars. Here we have shown that independent stimulation of electron transport (by expression of cytochrome c_6) and stimulation of RuBP regeneration (by expression of FBP/SBPase or overexpression of SBPase) increased photosynthesis and biomass in glasshouse studies. Furthermore, we demonstrated how the targeting of these two processes simultaneously (in the S_BC_6 and SC_6 plants) had an even greater effect in stimulating photosynthesis and growth. Additionally, in field studies we demonstrate that plants with simultaneous stimulation of electron transport and of RuBP regeneration had increased *iWUE* with an increase in biomass.

Under glasshouse conditions, increases in photosynthesis were observed in all of the transgenic plants analysed here and this was found to be correlated with increased biomass. Although increases in photosynthesis and biomass have been reported for plants with stimulation of RuBP regeneration in both model[4,5,8,7,27] and crop[18,16] species; and electron transport in Arabidopsis and tobacco[20,22,28], the data presented here provides the first report of increased photosynthesis and biomass by the simultaneous stimulation of electron transport and RuBP regeneration. Increases in A were observed under glasshouse conditions in the leaves of all of the different transgenic tobacco plants and in both tobacco cultivars (cv. Petit Havana and cv. Samsun). Analysis of the A/C_i response curves showed that the average values for the photosynthetic parameters Vc_{max}, J_{max} and A_{max} increased by up to 11, 14 and 15% respectively. These results indicated that not only was the maximal rate of electron transport and RuBP regeneration increased, but the rate of carboxylation by Rubisco was also increased. Although this may seem counterintuitive in that we have not targeted directly

Rubisco activity, it is in keeping with a study by Wullschleger[29] of over 100 plant species that showed a linear correlation between J_{max} and Vc_{max}. Furthermore, it has also been shown previously that overexpression of SBPase leads not only to a significant increase in J_{max} but that an increase in Vc_{max} and Rubisco activation state[5,8].

Notably, in the greenhouse study, the highest photosynthetic rates were observed in plants in which both electron transport and RuBP regeneration (S_BC_6 and SC_6) were boosted, suggesting that the co-expression of these genes results in an additive effect on improving photosynthesis. In addition to the increases in *A*, the plants with simultaneous stimulation of electron transport and RuBP regeneration displayed a significant increase in F_q'/F_m', indicating a higher quantum yield of linear electron flux through PSII compared to the control plants. These results are in keeping with the published data for the introduction of cytochrome c_6 and the overexpression of the Rieske FeS protein in Arabidopsis[20,22]. In these studies the plants had a higher quantum yield of PSII and a more oxidised plastoquinone pool[22], suggesting that, although PC is not always limiting under all growth conditions[30], there is scope to stimulate reduction of PSI by using alternative, more efficient electron donors to PSI like cytochrome c_6[22,26]. Furthermore, in the S_BC_6 and SC_6 plants the increase in F_q'/F_m' was found to be largely driven by the increase in the PSII efficiency factor (F_q'/F_v'). This suggests that the increase in efficiency in these plants is likely due to stimulation of processes down stream of PSII such as CO_2 assimilation.

To provide further evidence of the applicability of targeting both electron transport and RuBP regeneration to improve crop yields, we tested these plants in the field. Here we showed that the expression FBP/SBPase alone led to an increase in growth and biomass in the 2016 field-grown plants of between 22-40%, when harvested during early vegetative

279 growth, prior to the onset of flowing. Interestingly, when these plants were harvested later in
280 development, after the onset of flowering, in the 2017 field trials, this advantage was no
281 longer evident and the single FBP/SBPase expressors were indistinguishable from the control
282 plants. These results are in contrast to the 2016 field data and may be due to the later timing
283 in development of the harvest in the 2017 experiment. The transgenic plants expressing
284 cytochrome c_6 alone also showed enhanced growth and biomass early development, but as
285 with the FBPase/SBPase plants, this improvement was no longer evident when plants were
286 harvested after flowering. This difference in biomass gain between the early and late harvest
287 was not observed in a parallel experiment, where the overexpression of H-protein was shown
288 to increase biomass under field conditions in plants harvested in early development and after
289 the onset of flowering[31]. These results suggest that the expression of FBP/SBPase or
290 cytochrome c_6 alone, may provide an advantage under particular sets of conditions or at
291 specific stages of plant development. This might be exploitable for some crops where an
292 early harvest is desirable (eg. some types of lettuce, spinach and tender greens)[18]. In contrast
293 to the results with the single manipulations described above, plants expressing both
294 cytochrome c_6 and FBP/SBPase simultaneously displayed a consistent increase in biomass
295 after flowering under field conditions.
296
297 In the transgenic lines grown in the field, the correlation between increases in
298 photosynthesis and increased biomass were less consistent than that observed under
299 glasshouse conditions. The significant increases in photosynthetic capacity displayed by the
300 FBP/SBPase and cytochrome c_6 expressors in 2017 Experiment 1, provided clear evidence
301 that these individual manipulations are able to significantly stimulate photosynthetic
302 performance under field conditions. However, no increase in biomass was evident in these
303 plants. In contrast, in the 2017 Experiment 2 we did not detect any significant differences in

304 photosynthetic capacity in either the cytochrome c_6 expressors or the plants with
305 simultaneous expression of FBP/SBPase + cytochrome c_6 expressors, but increased biomass
306 was evident. At this point we have no explanation for this disparity. However, although not
307 significantly different, in all experiments, the mean *A* values of the transgenic plants were
308 consistently higher than those of the controls. It is known that even small increases in
309 assimilation throughout the lifetime of a plant will have a cumulative effect, which could
310 translate into a significant biomass accumulation[8], this may in part explain the disparity with
311 the biomass results presented. Furthermore, the phenotyping experiments carried out on C_6
312 and S_BC_6 plants (**Supplementary Fig 6**) showed that there was a more rapid induction of
313 photosynthesis, particularly in S_BC_6 plants. This characteristic might also contribute to an
314 increase in photosynthetic rates and biomass when plants are grown in fluctuating light
315 conditions, but would not be detectable in the steady-state measurements performed in our
316 field experiments.
317
318
319 An unexpected result that was found in the plants with simultaneous expression of
320 FBP/SBPase + cytochrome c_6 (S_BC_6), is that these plants had a lower g_s and lower C_i at light
321 intensities above 1000 µmol m^{-2} s^{-1}, when compared to control plants. Normally, lower C_i
322 would be expected to lead to a reduction in photosynthesis, but the S_BC_6 plants were able to
323 maintain CO_2 assimilation rates equal to or higher than control plants resulting in an
324 improvement in *iWUE*. A similar improvement in *iWUE* was seen in plants overexpressing
325 the NPQ related protein, PsbS[32]. It was shown that light-induced stomatal opening was
326 reduced in these plants in which a more oxidized Q_A pool was found and this has been
327 proposed to act as a signal in stomatal movement[33]. This higher *iWUE* and the fact that a
328 higher productivity than controls has been reported in field studies for transgenic lines with

329 increased RuBP regeneration grown under CO_2 enrichment[7,18], highlight the potential of
330 manipulating electron transport and RuBP regeneration in the development of new varieties
331 able to sustain photosynthesis and yields under climate change scenarios.

MATERIALS AND METHODS

Generation of constructs and transgenic plants

Constructs were generated using Golden Gate cloning[34,35] or Gateway cloning technology[36]. Transgenes were under the control of CaMV35S and FMV constitutive promoters. Construct detail below and in **Supplementary Fig. 12**.

For *N. tabacum* cv. Petit Havana, the codon optimised cyanobacterial bifunctional fructose-1,6-bisphosphatases/sedoheptulose-1,7-bisphosphatase (FBP/SBPase; *slr2094* Synechocystis sp. PCC 7942 [4] linked to the geraniol synthase transit peptide [37] and the codon optimised *P. umbilicalis's* cytochrome c_6 (AFC39870) with the chlorophyll a-b binding protein 6 transit peptide from Arabidopsis (AT3G54890) were used to generate Golden Gate[35] over-expression constructs (EC23083 and EC23028) driven by the FMV [38] and CaMV 35S promoters respectively (**Supplementary Fig. 12a**).

The cytochrome c_6 from *P. umbilicalis* was selected as it is commonly found on the UK coastline and it shares over 86% identity with previously published *P. yeoenzis* and *Ulva fasciata* used by Chida *et al* [22] and Yadav *et al* [23]. The level of similarity between these proteins and the fact that the functional regions are identical, provides confidence that the cytochrome c_6 proteins from these three species function in a similar way (see alignment in **Supplementary Fig. 13**). The *P. umbilicalis* cytochrome c_6 was linked to the transit peptide from the light-harvesting complex I chlorophyll a/b binding protein 6 (At3g54890) to generate an over-expression construct driven by the CaMV 35S promoter; B2-C6 in the vector pGWB2[36] used for *N. tabacum* cv. Samsun transformation (**Supplementary Fig. 12b**). The recombinant plasmid B2-C6, was introduced into SBPase over-expressing tobacco cv. Samsun[5] using *Agrobacterium tumefaciens* AGL1 via leaf-disc transformation[39]. Primary transformants (39) (T0 generation) were regenerated on MS medium containing kanamycin

(100 mg L^{-1}), hygromycin (30 mg L^{-1}) and augmentin (500 mg L^{-1}). Plants expressing the integrated transgenes were screened using RT-PCR (data not shown).

Similarly, the recombinant plasmids EC23083, and EC23028 were introduced into wild type tobacco (*Nicotiana tabacum*) cv Petit Havana, using *A. tumefaciens* strain LBA4404 via leaf-disc transformation[39], and shoots regenerated on MS medium containing, hygromycin (20 mg L^{-1}) and cefotaxime (400 mg L^{-1}). Hygromycin resistant primary transformants (T0 generation) with established root systems were transferred to soil and allowed to self-fertilize.

Between twelve and 60 independent lines were generated per construct and 3-4 lines taken forward for further analysis. Control (CN) plants used in this study were a combination of WT and null segregant plants from the transgenic lines, verified by PCR for non-integration of the transgene.

Plant Growth

Controlled conditions

Wild-type tobacco plants and T1 progeny resulting from self-fertilization of transgenic plants were grown to seed in soil (Levington F2, Fisons, Ipswich, UK). Lines of interest were identified by immunoblot and qPCR. For the experiments in the Samsun cv. the null segregants were selected from transformed lines. For Petit Havana, the null segregants were selected from the S_BC_6 lines. For experimental study, T2-T4 and F1-F3 progeny seeds were germinated on soil in controlled environment chambers at an irradiance of 130 µmol photons m^{-2} s^{-1}, 22°C, relative humidity of 60%, in a 16-h photoperiod. Plants were transferred to individual 8 cm pots and grown for two weeks at 130 µmol photons m^{-2} s^{-1}, 22°C, relative humidity of 60%, in a 16-h photoperiod. Plants were transferred to 4 L pots and cultivated in a controlled environment glasshouse (16-h photoperiod, 25°C-30°C

day/20°C night, and natural light supplemented under low light induced by cloud cover with high-pressure sodium light bulbs, giving 380-1000 µmol m^{-2} s^{-1} (high-light) from the pot level to the top of the plant, respectively). Positions of the plants were changed 3 times a week and watered regularly with a nutrient medium[40]. Plants were positioned such that at maturity, a near-to-closed canopy was achieved and the temperature range was maintained similar to the ambient external environment. Four leaf discs (0.8 cm diameter) were taken for immunoblot analysis and FBPase activity. These disks were taken from the same areas of the leaf used for photosynthetic measurements, immediately plunged into liquid N_2 and stored at -80°C.

Field studies

Plants were grown as described in Lopez-Calcagno et al[31], and with a methodology broadly analogous to that used commercially for this crop. The field site was situated at the University of Illinois Energy Farm (40.11°N, 88.21°W, Urbana, IL). Two different experimental designs were used in 2 different years.

2016: Replicated control design (**Supplementary Fig. 14a**). Plants were grown in rows, spaced 30 cm apart with the outer boundary being a wild-type border. The entire experiment was surrounded by two rows of wild-type borders. Plants were irrigated when required using rain towers. T2 seed was germinated and after 11 d were moved to individual pots (350 mL). The seedlings were grown in the glasshouse for further 15 d before being moved into the field, and allowed to grow in the field for 14 d before harvest.

2017: Two experiments were carried out two weeks apart. A blocks-within-rows design was used (**Supplementary Fig. 14b**) where 1 block holds one line of each of the five manipulations and each row has all lines. The central 20 plants of each block are divided into five rows of four plants per genotype. The 2017 Exp.1 contained controls (WT and null

segregants), FBP/SBPase expressing lines (S_B) and cytochrome c_6 expressing lines (C_6). The 2017 Exp. 2 contained controls (WT and null segregants), cytochrome c_6 expressing lines (C_6), and FBP/SBPase + cytochrome c_6 expressing lines (S_BC_6). Seed was germinated and after 12 d moved to hydroponic trays (Trans-plant Tray GP009 6912 cells; Speedling Inc., Ruskin, FL), and grown in the glasshouse for 20 d before being moved to the field. The plants were allowed to grow in the field until flowering (approximately 30 d) before harvest.

The field was prepared in a similar fashion each year as described in Kromdijk *et al*[41]. Light intensity (LI-quantum sensor; LI-COR) and air temperature (Model 109 temperature probe; Campbell ScientificInc, Logan, UT) were measured nearby on the same field site, and half-hourly averages were logged using a data logger (CR1000; Campbell Scientific).

cDNA generation and RT-PCR

Total RNA was extracted from tobacco leaf disks (sampled from glasshouse grown plants and quickly frozen in liquid nitrogen) using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Fisher Scientific, UK). cDNA was synthesized using 1 µg total RNA in 20 µl using the oligo-dT primer according to the protocol in the RevertAid Reverse Transcriptase kit (Fermentas, Life Sciences, UK). cDNA was diluted 1 in 4 to a final concentration of 12.5 ng μL^{-1}. For semi quantitative RT-PCR, 2 µL of RT reaction mixture (100 ng of RNA) in a total volume of 25 µL was used with DreamTaq DNA Polymerase (Thermo Fisher Scientific, UK) according to manufacturer's recommendations. PCR products were fractionated on 1.0% agarose gels. For qPCR, the SensiFAST SYBR No-ROX Kit was used according to manufacturer's recommendations (Bioline Reagents Ltd., London, UK). Primers used for semi quantitative RT-PCR can be seen in **Supplementary Table 1.**

Protein Extraction and immunoblot analysis

Leaf discs sampled as described above, or fresh *Porphyra umbilicalis* samples, were ground in dry ice and protein extractions performed as described in Lopez-Calcagno *et al.*[42], or using the nucleospin RNA/Protein kit (Macherey-Nagel (http://www.mn-net.com/) during RNA preparations. Protein quantification was performed using the protein quantification Kit from Macherey-Nagel. Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare Life science, Germany), and probed using antibodies raised against SBPase and FBP/SBPase. Proteins were detected using horseradish peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham, Buckinghamshire, UK). SBPase antibodies are previously characterised[5,43]. FBP/SBPase antibodies were raised against a peptide from a conserved region of the protein [C]-DRPRHKELIQEIRNAG-amide, and cytochrome c_6 antibodies were raised against peptide [C]-[Nle]-PDKTLKKDVLEANS-amide (Cambridge Research Biochemicals, Cleveland, UK). In addition to the aforementioned antibodies, samples were probed using antibodies raised against transketolase[44,45] as loading controls.

Protein Extraction from plants for cytochrome c_6 analysis.

Whole leaves were harvested from 8 week old plants, washed in cold water and then wiped with a cloth soaked in 80 % ethanol to remove the majority of leaf residue. The leaves were then washed twice more in cold water, the mid rib was removed and 50 g of the remaining tissue was placed in a sealed plastic bag and stored overnight in the dark at 4˚C. Proteins were extracted as in Hiyama[47], with a few modifications. Leaf tissue was homogenised in 250 ml of chilled chloroplast preparation buffer (50 mM sodium phosphate buffer, pH 7, 10 mM NaCl) for 30 seconds. The solution was then filtered through 4 layers of muslin cloth and centrifuged at 10,000 g for 5 minutes. The resulting pellet was then gently

457 resuspended in 50 ml of chilled chloroplast preparation buffer and the chlorophyll
458 concentration was measured and adjusted to approximately 2 mg ml^{-1}. The resultant mixture
459 was then added to two volumes of preheated (45˚C) solubilisation medium (50 mM Tris-HCl
460 pH 8.8 and 3% triton X) and incubated at 45˚C for 30 minutes and then chilled in an ice bath
461 for a further 30 minutes before centrifugation at 12000 g for 30 minutes. The supernatant was
462 stored at -80˚C for use in the next stage. To purify cytochrome c_6 protein a Biorad Econo-Pac
463 High-Q, 5 ml type wash column was used at a flow rate of 1 ml min^{-1}. First the column was
464 prepared by washing it with 100 ml of starting buffer (Starting buffer: 10 mM Tris-HCl pH
465 8.8, 0.2% triton X 100 and 20% sucrose). Then the protein mixture from the previous step
466 was diluted with an equal volume of chilled starting buffer and passed through the column at
467 a flow rate of 1 ml min^{-1}. Once all the protein was loaded onto the column it was then washed
468 with 1000 ml of starting buffer supplemented with 10 mM NaCl. Then 300 ml of starting
469 buffer supplemented with 50 ml NaCl and finally a linear gradient of starting buffer from 50
470 to 200 mM NaCl over a period of 4 hours at 1 ml min^{-1} was performed and aliquots were
471 collected. For immunoblotting, samples were acetone precipitated and the dried protein pellet
472 then resuspended in 400 µl of solubilisation buffer (7 M urea, 2 M thiourea, 50 mM DTT, 4
473 % CHAPS, 0.4 % SDS, 5 mM K_2CO_3), finally 300 µl loading buffer was added (50%
474 glycerol, 25% β-mecaptoethanol, 25% EDTA) and the samples heated at 90˚C for 10 minutes
475 before being loaded on an equal protein basis. Proteins were separated using 18% (w/v) SDS-
476 PAGE, transferred to nitrocellulose membrane, and probed using antibodies raised against a
477 cytochrome c_6 peptide. For identification of soret peak, instead of acetone precipitation,
478 extracts were concentrated by spinning at 8,000 g and 4˚C over night, using a Vivaspin 20
479 column (GE 28-9323-59), and a spectral scan was done in a SPECTROstar Omega plate
480 reader from BMG Labtech.
481

Recombinant cytochrome c_6 protein production in *E. coli* and purification

pEC86 (CCOS Accession: CCOS891) containing *E. coli* cells were transformed with a pET28b plasmid containing the sequence for the mature cytochrome c_6 and grown in kanamycin (50 ug/ml) and chloramphenicol (35ug/ml) containing LB media. IPTG (119 µg ml^{-1}) was added to the culture when OD_{600} reached 0.5-0.6. Five hours later 330 µl L^{-1} of 1 M ferriprotoporphyrin IX chloride was added to the media and 24 hours post IPTG, an metal ion master mix (2 mM Ni^{2+}, 2 mM Co^{2+}, 10mM Zn^{2+}, 10 mM Mn^{2+} and 50 mM Fe^{3+}) was added (1.5 ml L^{-1}). Cells were harvested after 5 days of growth and stored at -20 ˚C. Pellet from 500 ml was resuspended in 3 ml of lysis buffer (50mM Tris HCl pH 7.5, 1mM DTT, 1mM PMSF), sonicated (11 cycles of 30 sec sonication 30 sec rest, at 4˚C) and then spun twice at 10000 g for 20 min at 4 ˚C. The supernatant was collected and 2 ml loaded in a 124 ml GE Hi Load 16/400 Superdex 75 pg (size exclusion) column. Protein was eluted with 0.05 M Na_2PO_4 pH 7.2, 0.5 M NaCl buffer, at a 1 ml min^{-1} speed and samples were collected every 5 ml. Fractions collected between 80-100 min were concentrated by spinning them at 8000 g over night at 4 ˚C using a Vivaspin 20, (GE 28-9323-59) column. Protein concentration was determined using Bradford quantification, serial dilutions done with 50 mM Tris HCl pH 7.5 buffer and spectral scans done in a SPECTROstar Omega plate reader from BMG Labtech as with the semi-purified plant cytochrome c_6 samples.

Determination of FBPase and Transketolase Activities

FBPase activity was determined by phosphate release as described previously for SBPase with minor modifications[8]. Leaf discs were isolated from the same leaves and frozen in liquid nitrogen after photosynthesis measurements were completed. Leaf discs were ground to a fine powder in liquid nitrogen and immersed in extraction buffer (50 mM HEPES, pH8.2; 5 mM MgCl; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.1% Triton X-

100; 2 mM benzamidine; 2 mM aminocapronic acid; 0.5 mM phenylmethylsulfonylfluoride; 10 mM dithiothreitol), centrifuged 1 min at 14,000 g, 4°C. The resulting supernatant (1 ml) was desalted through an NAP-10 column (Amersham) and stored in liquid nitrogen. The assay was carried out as descried in Simkin *et al.*[8]. In brief, 20 µl of extract was added to 80 µl of assay buffer (50 mM Tris, pH 8.2; 15 mM $MgCl_2$; 1.5 mM EDTA; 10 mM DTT; 7.5 mM fructose-1,6-bisphosphate) and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 50 µl of 1 M perchloric acid. 30 µl of samples or standards (PO^{3-}_4 0.125 to 4 nmol) were incubated 30 min at room temperature following the addition of 300 µl of Biomol Green (Affiniti Research Products, Exeter, UK) and the A620 was measured using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA). Activities were normalized to transketolase activity[48]. For transketolase activity assays 230 µl of pre-prepared assay mix comprising of: 14.4 Mm ribose-5-phosphate, 190 µM NADH, 380 µM TPP, 250 U L^{-1} glycerol-3 phosphate dehydrogenase (G3PDH) and 6500 U L^{-1} triose phosphate isomerase was transferred to a 96 well plate (Greiner Bio-One) and placed in a plate reader which was set at 23 ˚C for 5 minutes to stabilise. The plate was then ejected and 20 µl of each protein sample used for FBPase activity was injected into the wells containing the assay mix. The plate was then read for absorbance at 340 nm every 5 min for 1 hr. Activity levels were estimated by subtracting the absorbance value when the reaction becomes linear from the absorbance value 20 to 30 minutes after the first absorbance reading depending on the rate of the reaction.

Chlorophyll fluorescence imaging screening in seedlings

Chlorophyll fluorescence imaging was performed on 2-3 week-old tobacco seedlings grown in a controlled environment chamber at 130 µmol mol^{-2} s^{-1} and ambient (400 µmol mol^{-1}) CO_2. Chlorophyll fluorescence parameters were obtained using a chlorophyll

fluorescence (CF) imaging system (Technologica, Colchester, UK[49,50]). The operating efficiency of photosystem two (PSII) photochemistry, F_q'/F_m', was calculated from measurements of steady state fluorescence in the light (F') and maximum fluorescence (F_m') following a saturating 800 ms pulse of 6300 µmol m^{-2} s^{-1} PPFD and using the following equation $F_q'/F_m' = (F_m'-F')/F_m'$. Images of F_q'/F_m' were taken under stable PPFD of 600 µmol m^{-2} s^{-1} for Petite Havana and 650 µmol m^{-2} s^{-1} for Samsun[51-53].

Leaf Gas Exchange

Photosynthetic gas-exchange and chlorophyll fluorescence parameters were recorded using a portable infrared gas analyser (LI-COR 6400; LI-COR, Lincoln, NE, USA) with a 6400-40 fluorometer head unit. Unless stated otherwise, all measurements were taken with LI-COR 6400 cuvette. For plants grown in the glasshouse conditions were maintained at a CO_2 concentration, leaf temperature and vapour pressure deficit (VPD) of 400 µmol mol^{-1}, 25 ˚C and 1 ± 0.2 kPa respectively. The chamber conditions for plants grown under field conditions had a CO_2 concentration of 400 µmol mol^{-1}, block temperature was set to 2 ˚C above ambient temperature (ambient air temperature was measure before each curve) and VPD was maintained as close to 1 kPa as feasible possible.

***A/C*$_i$ response curves (Photosynthetic capacity)**

The response of net photosynthesis (A) to intracellular CO_2 concentration (C_i) was measured at a saturating light intensity of 2000 µmol mol^{-2} s^{-1}. Illumination was provided by a red-blue light source attached to the leaf cuvette. Measurements of A were started at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1}, before C_a was decreased step-wise to a lowest concentration of 50 µmol mol^{-1} and then increased step-wise to an upper concentration of 2000 µmol mol^{-1}. To calculate the maximum saturated CO_2 assimilation rate (A_{max}),

maximum carboxylation rate (Vc_{max}) and maximum electron transport flow (J_{max}), the C3 photosynthesis model[54] was fitted to the A/C_i data using a spreadsheet provided by Sharkey *et al.*[55]. Additionally, chlorophyll fluorescence parameters including PSII operating efficiency (F_q'/F_m') and the coefficient of photochemical quenching (q_P), mathematically identical to the PSII efficiency factor (F_q'/F_v') were recorded at each point.

***A/Q* response curves**

Photosynthesis as a function of light (*A*/*Q* response curves) was measured under the same cuvette conditions as the A/C_i curves mentioned above. Leaves were initially stabilized at saturating irradiance of 2200 to µmol m^{-2} s^{-1}, after which *A* and g_s were measured at the following light levels: 2000, 1650, 1300, 1000, 750, 500, 400, 300, 200, 150, 100, 50 and 0 µmol m^{-2} s^{-1}). Measurements were recorded after *A* reached a new steady state (1-3 min) and before g_s changed to the new light levels. Values of *A* and g_s were used to estimate intrinsic water-use efficiency ($iWUE = A/g_s$)

Monitoring electron transport and assimilation during light changes.

A DUAL-PAM attached to a GFS-3000 (Walz, Effeltrich, Germany) was used to monitor the response of the effective photochemical quantum yield of PSII (F_q'/F_m') and PSI (Y(I)), and the net CO_2 Assimilation (*A*) to changes in light intensity. To remove stomatal limitation of *A*, plants were maintained at constant temperature (24°C), relative humidity (60%) and high [CO_2] (1500 µmol mol^{-1}). Plants were dark adapted and the induction/relaxation of the photosystems was tested by subjecting plants to a step change in light intensity from 0 to 1000 µmol m^{-2} s^{-1}, this intensity was maintained for 5 min before returning to dark.

582 **Statistical Analysis**

583 All statistical analyses were done using Sys-stat, University of Essex, UK, and R
584 (https://www.r-project.org/). For greenhouse and the 2016 field experiment biomass data,
585 seedling chlorophyll imaging and enzyme activities, analysis of variance and Post hoc Tukey
586 tests were done. For gas exchange curves, data were compared by linear mixed model
587 analysis using lmer function and type III anova[56]. Significant differences between
588 manipulations were identified using contrasts analysis (lsmeans package). For the 2017 field
589 experiments, biomass data were compared by linear mixed model analysis using lmer
590 function and type III anova to account for block effect using four plants/genotype for n=6
591 blocks. For the analysis of electron transport and assimilation during light changes, the slope
592 of the activation curves was calculated for each parameter and analysis of variance and post-
593 hoc Tukey test was done.

594

595 **Data availability**

596 The data that support the findings of this study, plant transformation constructs and
597 seed are available from the corresponding authors on reasonable request.

598 **Figure Legends**

599

600 **Fig. 1. Screening of transgenic plants overexpressing FBP/SBPase, SBPase, and**
601 **cytochrome c_6.**

602 (a) Immunoblot analysis of protein extracts from mature leaves of evaluated S_B , S_BC_6, S and
603 SC_6 lines compared to wild type and azygous (control, CN) plants, using FBP/SBPase and
604 SBPase antibodies. Equal amounts of protein were loaded, Transketolase (TK) is the loading
605 control. Repeated 3 times with similar results. (b) Immunoblot analysis of Cytochrome c_6
606 protein extract from mature leaves of C_6 compared to CN plants, ponceau staining was used
607 as loading control for plant samples only. Additionally, a crude *Porphyra sp.* protein extract is
608 presented as confirmation of correct band size for the introduced Cytochrome c_6. Repeated 3
609 times with similar results (c) FBPase activity in S_B (n=16) and S_BC_6 (n=14) relative to CN
610 (n=6) plants. Chlorophyll fluorescence imaging of plants grown in controlled environmental
611 conditions was used to determine F_q'/F_m' (maximum PSII operating efficiency) at 600-650
612 µmol m^{-2} s^{-1}, 14 to 21 days after sowing (d) CN (n=20), S_B (n= 28), C_6 (n=29), S_BC_6 (n=30),
613 (e) CN (n=11), S (n=7) and SC_6 (n=6). Mean and SE is presented. Statistical tests used
614 analysis of variance and post-hoc Tukey test.

615

616 **Fig 2. Photosynthetic responses of transgenic plants grown in the glasshouse.**

617 Photosynthetic carbon fixation rates, operating efficiency of PSII in the light (F_q'/F_m'), PSII
618 efficiency factor (F_q'/F_v') and PSII maximum efficiency (F_v'/F_m') are presented in (a) mature
619 leaves CN (n=10), S_B (n=7), C_6 (n=11), S_BC_6 (n=9) cv. Petit Havana (b) mature leaves of CN
620 (n=10), S (n=8), SC_6 (n=10) and (c) developing leaves CN (n=6), S (n=6), SC_6 (n=9) cv.

Samsun. Parameters were determined as a function of increasing CO_2 concentrations at saturating-light levels in developing (11-13cm in length) and mature leaves. Plants were grown in the glasshouse where light levels oscillated between 400 and 1000 μmol m^{-2} s^{-1} (supplemental light ensured a minimum of 400 μmol m^{-2} s^{-1}). Control group (CN) represent both WT and azygous plants. Asterisks indicate significance between the transgenics and CN plants, using a linear mixed-effects model and type III ANOVA and contrast analysis, *p < 0.05, exact p value indicated in each plot.

Figure 3. Increased SBPase or expression of FBP/SBPase and cytochrome c_6 increases biomass in glasshouse grown plants.

Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination. Forty-day-old (cv. Petit Havana) or fifty-six-day-old (cv. Samsun) plants were harvested and plant height, leaf area and above-ground biomass (dry weight) determined. Control group represent both WT and azygous plants (CN). cv. Petite Havana CN (n=17), S_B (n=21), C_6 (n=18), (S_BC_6 n=18); cv. Samsun CN (n= 16), S (n=7, SC_6 (n= 13). Mean and SE is presented. Statistical analysis was ANOVA with post-hoc Tukey test.

Figure 4. Simultaneous expression of FBP/SBPase and cytochrome c_6 increases biomass in field grown plants.

(a-c) Forty-day-old (young) 2016 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and then allowed to grow in the field in summer 2016 for 14 d); (d-i) Fifty-seven-day-old or sixty-one-day-old (flowering) 2017 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and grown in the field in summer 2017 until flowering established, circa 30 d). Plant height, leaf area and total above-ground biomass (dry weight) are shown. 2016 Experiment CN (n=72), S_B (n=33), C_6 (n=33);

2017 Experiment 1: CN (n=93), S_B (n=71), C_6 (n=70); 2017 Experiment 2: (n=97), C_6 (n=72), S_BC_6 (n=47) Mean ± SE presented. Statistical analysis was ANOVA with post-hoc Tukey test.

Fig 5. Photosynthetic capacity of field-grown transgenic plants.

Photosynthetic carbon fixation rates and operating efficiency of PSII as a function of increasing CO_2 concentrations at saturating-light levels in mature leaves from CN and transgenic plants. (a) 2017 experiment 1: CN (n= 21), S_B (n=16) and C_6 (n=16). (b) 2017 experiment 2: Lines expressing cytochrome CN (n=22) C_6 (n=16), S_BC_6 (n=14). Control group (CN) represent both WT and azygous plants. Mean ± SE presented. A linear mixed-effects model and type III ANOVA was applied, exact p value indicated in each plot.

Fig 6. Simultaneous expression of FBP/SBPase and cytochrome c_6 can increase water use efficiency under field conditions.

(a) Net CO_2 assimilation rate (A), (b) Stomatal conductance (g_s), (c) Intercellular CO_2 concentration (C_i), and (d) Intrinsic water-use efficiency (iWUE) as a function of light (PPFD) in field-grown plants, CN n= 22, C_6 n=16, S_BC_6 n=14. A linear mixed-effects model and type III ANOVA was applied, exact p value indicated in each plot.

Table 1. Maximum electron transport and RuBP regeneration rate (J_{max}), maximum carboxylation rate of Rubisco (Vc_{max}) and maximum assimilation (A_{max}) of WT and transgenic lines. Results were determined from the A/C_i curves in Figure 2 using the equations published by von Caemmerer and Farquhar[57]. Significant differences are shown in boldface (* $p<0.05$). cv. Samsun Mature leaves CN (n=10), S (n=8), SC_6 (n=10); developing leaves CN (n=6), S (n=6), SC_6 (n=9); cv. Petit Havana Mature leaves: CN (n=10), S_B (n=7), C_6 (n=11), S_BC_6 (n=9) Mean and SE are shown.

			A/C_i		
	Leaf Stage	Line	Vc_{max} (µmol m^{-2} s^{-1})	J_{max} (µmol m^{-2} s^{-1})	A_{max} (µmol m^{-2} s^{-1})
Samsun	Developing	CN	72.32 ± 5.5	157.51 ± 6.0	29.6 ± 1.1
		S	87.7 ± 4.3	**179.8± 4.9***	**34.1 ± 0.7***
		SC_6	86.5 ± 3.5	**181.2 ± 3.6***	**33.7 ± 1.1***
	Mature	CN	77.2 ± 3.3	171.0 ± 6.0	31.6 ± 1.0
		S	81.3 ± 6.1	183.5 ± 9.0	32.2 ± 0.7
		SC_6	90.3 ± 3.3	193.1 ± 5.4	**34.9 ± 1.1***
Petit Havana	Mature	CN	69.6 ± 2.0	121.5 ± 1.3	24.6 ± 0.5
		S_B	69.0 ± 5.1	128.7 ± 3.8	27.0 ± 0.8
		C_6	79.3 ± 7.0	129.9 ± 5.1	25.6 ± 0.5
		S_BC_6	76.5 ± 4.2	132.0 ± 3.8	**27.4 ± 0.8***

References

1 Zhu, X. G., Long, S. P. & Ort, D. R. Improving photosynthetic efficiency for greater yield. *Annu Rev Plant Biol* **61**, 235-261, doi:10.1146/annurev-arplant-042809-112206 (2010).

2 Simkin, A. J., Lopez-Calcagno, P. E. & Raines, C. A. Feeding the world: Improving photosynthetic efficiency for sustainable crop production. *Journal of Experimental Botany*, doi:doi.org/10.1093/jxb/ery445 (2019).

3 Simkin, A. J. Genetic Engineering for Global Food Security: Photosynthesis and Biofortification. *Plants* **8**, 586 (2019).

4 Miyagawa, Y., Tamoi, M. & Shigeoka, S. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nat Biotechnol* **19**, 965-969, doi:10.1038/nbt1001-965 (2001).

5 Lefebvre, S. *et al.* Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant physiology* **138**, 451-460, doi:10.1104/pp.104.055046 (2005).

6 Raines, C. A. Transgenic approaches to manipulate the environmental responses of the C(3) carbon fixation cycle. *Plant Cell Environ* **29**, 331-339, doi:10.1111/j.1365-3040.2005.01488.x (2006).

7 Rosenthal, D. M. *et al.* Over-expressing the C(3) photosynthesis cycle enzyme Sedoheptulose-1-7 Bisphosphatase improves photosynthetic carbon gain and yield under fully open air CO(2) fumigation (FACE). *BMC Plant Biol* **11**, 123, doi:10.1186/1471-2229-11-123 (2011).

8 Simkin, A. J., McAusland, L., Headland, L. R., Lawson, T. & Raines, C. A. Multigene manipulation of photosynthetic carbon assimilation increases CO_2 fixation and biomass yield in tobacco. *J Exp Bot* **66**, 4075-4090, doi:10.1093/jxb/erv204 (2015).

9 Simkin, A. J. *et al.* Simultaneous stimulation of sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphophate aldolase and the photorespiratory glycine decarboxylase-H protein increases CO2 assimilation, vegetative biomass and seed yield in Arabidopsis. *Plant Biotechnol J* **15**, 805-816, doi:10.1111/pbi.12676 (2017).

10 Zhu, X. G., de Sturler, E. & Long, S. P. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: A numerical simulation using an evolutionary algorithm. *Plant physiology* **145**, 513-526, doi:DOI 10.1104/pp.107.103713 (2007).

11 Long, S. P., Zhu, X. G., Naidu, S. L. & Ort, D. R. Can improvement in photosynthesis increase crop yields? *Plant Cell Environ* **29**, 315-330 (2006).

12 Poolman, M. G., Fell, D. A. & Thomas, S. Modelling photosynthesis and its control. *J Exp Bot* **51**, 319-328, doi:DOI 10.1093/jexbot/51.suppl_1.319 (2000).

13 Raines, C. A. The Calvin cycle revisited. *Photosynth Res* **75**, 1-10, doi:Doi 10.1023/A:1022421515027 (2003).

14 Uematsu, K., Suzuki, N., Iwamae, T., Inui, M. & Yukawa, H. Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants. *J Exp Bot* **63**, 3001-3009, doi:10.1093/jxb/ers004 (2012).

15 Ding, F., Wang, M. L., Zhang, S. X. & Ai, X. Z. Changes in SBPase activity influence photosynthetic capacity, growth, and tolerance to chilling stress in transgenic tomato plants. *Sci Rep-Uk* **6**, doi:ARTN 32741
10.1038/srep32741 (2016).

16 Driever, S. M. *et al.* Increased SBPase activity improves photosynthesis and grain yield in wheat grown in greenhouse conditions. *Philosophical Transactions of the Royal Society B* **372**, 1730 (2017).
17 Tamoi, M., Nagaoka, M., Miyagawa, Y. & Shigeoka, S. Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. *Plant Cell Physiology* **47**, 380-390, doi:10.1093/pcp/pcj004 (2006).
18 Ichikawa, Y. *et al.* Generation of transplastomic lettuce with enhanced growth and high yield. *GM Crops* **1**, 322-326, doi:10.4161/gmcr.1.5.14706 (2010).
19 Kohler, I. H. *et al.* Expression of cyanobacterial FBP/SBPase in soybean prevents yield depression under future climate conditions. *J Exp Bot* **68**, 715-726, doi:10.1093/jxb/erw435 (2017).
20 Simkin, A. J., McAusland, L., Lawson, T. & Raines, C. A. Overexpression of the RieskeFeS Protein Increases Electron Transport Rates and Biomass Yield. *Plant Physiol* **175**, 134-145, doi:10.1104/pp.17.00622 (2017).
21 Ermakova, M., Lopez-Calcagno, P. E., Raines, C. A., Furbank, R. T. & von Caemmerer, S. Overexpression of the Rieske FeS protein of the Cytochrome b_6f complex increases C4 photosynthesis in *Setaria viridis*. *Communications Biology* **2**, 314, doi:10.1038/s42003-019-0561-9 (2019).
22 Chida, H. *et al.* Expression of the algal cytochrome c_6 gene in Arabidopsis enhances photosynthesis and growth. *Plant & cell physiology* **48**, 948-957, doi:10.1093/pcp/pcm064 (2007).
23 Yadav, S. K., Khatri, K., Rathore, M. S. & Jha, B. Introgression of UfCyt c_6, a thylakoid lumen protein from a green seaweed *Ulva fasciata* Delile enhanced photosynthesis and growth in tobacco. *Molecular biology reports* **45(6)**, 1745-1758, doi:10.1007/s11033-018-4318-1 (2018).
24 Merchant, S. & Bogorad, L. The Cu(II)-repressible plastidic cytochrome *c*. Cloning and sequence of a complementary DNA for the pre-apoprotein. *Journal of Biological Chemistry* **262**, 9062-9067 (1987).
25 De la Rosa M.A., M.-H. F. P., Hervás M., Navarro J.A. Convergent Evolution of Cytochrome c6 and Plastocyanin. *In: Golbeck J.H. (eds) Photosystem I. Advances in Photosynthesis and Respiration, vol 24. Springer, Dordrecht* (2006).
26 Finazzi, G., Sommer, F. & Hippler, M. Release of oxidized plastocyanin from photosystem I limits electron transfer between photosystem I and cytochrome b6f complex in vivo. *Proc Natl Acad Sci U S A* **102**, 7031-7036, doi:10.1073/pnas.0406288102 (2005).
27 Gong, H. Y. *et al.* Transgenic Rice Expressing Ictb and FBP/Sbpase Derived from Cyanobacteria Exhibits Enhanced Photosynthesis and Mesophyll Conductance to CO2. *Plos One* **10**, doi:ARTN e0140928
10.1371/journal.pone.0140928 (2015).
28 Yadav, S. K., Khatri, K., Rathore, M. S. & Jha, B. Introgression of UfCyt c6, a thylakoid lumen protein from a green seaweed Ulva fasciata Delile enhanced photosynthesis and growth in tobacco. *Mol Biol Rep*, doi:10.1007/s11033-018-4318-1 (2018).
29 Wullschleger, S. D. Biochemical Limitations to Carbon Assimilation in C(3) Plants - a Retrospective Analysis of the A/Ci Curves from 109 Species. *J Exp Bot* **44**, 907-920, doi:DOI 10.1093/jxb/44.5.907 (1993).
30 Pesaresi, P. *et al.* Mutants, Overexpressors, and Interactors of Arabidopsis Plastocyanin Isoforms: Revised Roles of Plastocyanin in Photosynthetic Electron

772 Flow and Thylakoid Redox State. *Mol Plant* **2**, 236-248, doi:10.1093/mp/ssn041
773 (2009).
774 31 López-Calcagno, P. E. *et al.* Overexpressing the H□protein of the glycine cleavage
775 system increases biomass yield in glasshouse and field□grown transgenic tobacco
776 plants. *Plant biotechnology journal* **17**, 141-151, doi:doi.org/10.1111/pbi.12953
777 (2018).
778 32 Glowacka, K. *et al.* Photosystem II Subunit S overexpression increases the efficiency
779 of water use in a field-grown crop. *Nat Commun* **9**, doi:ARTN 868
780 10.1038/s41467-018-03231-x (2018).
781 33 Busch, F. A. Opinion: The red-light response of stomatal movement is sensed by the
782 redox state of the photosynthetic electron transport chain. *Photosynth Res* **119**, 131-
783 140, doi:10.1007/s11120-013-9805-6 (2014).
784 34 Engler, C., Gruetzner, R., Kandzia, R. & Marillonnet, S. Golden Gate Shuffling: A
785 One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. *Plos One* **4**,
786 doi:ARTN e5553
787 10.1371/journal.pone.0005553 (2009).
788 35 Engler, C., Kandzia, R. & Marillonnet, S. A one pot, one step, precision cloning
789 method with high throughput capability. *Plos One* **3**, e3647,
790 doi:10.1371/journal.pone.0003647 (2008).
791 36 Nakagawa, T. *et al.* Development of series of gateway binary vectors, pGWBs, for
792 realizing efficient construction of fusion genes for plant transformation. *Journal of*
793 *bioscience and bioengineering* **104**, 34-41, doi:10.1263/jbb.104.34 (2007).
794 37 Simkin, A. J. *et al.* Characterization of the plastidial geraniol synthase from
795 *Madagascar periwinkle* which initiates the monoterpenoid branch of the alkaloid
796 pathway in internal phloem associated parenchyma. *Phytochemistry* **85**, 36-43,
797 doi:10.1016/j.phytochem.2012.09.014 (2013).
798 38 Richins, R. D., Scholthof, H. B. & Shepherd, R. J. Sequence of Figwort Mosaic-Virus
799 DNA (Caulimovirus Group). *Nucleic acids research* **15**, 8451-8466, doi:DOI
800 10.1093/nar/15.20.8451 (1987).
801 39 Horsch, R. B., Rogers, S. G. & Fraley, R. T. Transgenic Plants - Technology and
802 Applications. *Abstr Pap Am Chem S* **190**, 67 (1985).
803 40 Hoagland, D. R. & Arnon, D. I. *The water-culture method for growing plants without*
804 *soil*. (The College of Agriculture, 1950).
805 41 Kromdijk, J. *et al.* Improving photosynthesis and crop productivity by accelerating
806 recovery from photoprotection. *Science* **354**, 857-861, doi:10.1126/science.aai8878
807 (2016).
808 42 Lopez-Calcagno, P. E., Abuzaid, A. O., Lawson, T. & Raines, C. A. Arabidopsis
809 CP12 mutants have reduced levels of phosphoribulokinase and impaired function of
810 the Calvin-Benson cycle. *J Exp Bot* **68**, 2285-2298, doi:10.1093/jxb/erx084 (2017).
811 43 Dunford, R. P., Catley, M. A., Raines, C. A., Lloyd, J. C. & Dyer, T. A. Purification
812 of active chloroplast sedoheptulose-1,7-bisphosphatase expressed in Escherichia coli.
813 *Protein expression and purification* **14**, 139-145, doi:10.1006/prep.1998.0939 (1998).
814 44 Henkes, S., Sonnewald, U., Badur, R., Flachmann, R. & Stitt, M. A small decrease of
815 plastid transketolase activity in antisense tobacco transformants has dramatic effects
816 on photosynthesis and phenylpropanoid metabolism. *The Plant cell* **13**, 535-551
817 (2001).
818 45 Khozaei, M. *et al.* Overexpression of plastid transketolase in tobacco results in a
819 thiamine auxotrophic phenotype. *The Plant cell* **27**, 432-447,
820 doi:10.1105/tpc.114.131011 (2015).

46 Timm, S. *et al.* Glycine decarboxylase controls photosynthesis and plant growth. *FEBS letters* **586**, 3692-3697, doi:10.1016/j.febslet.2012.08.027 (2012).

47 Hiyama, T. Isolation of photosystem I particles from spinach. *Methods Mol Biol* **274**, 11-17, doi:10.1385/1-59259-799-8:011 (2004).

48 Zhao, Y. L. *et al.* Downregulation of Transketolase Activity Is Related to Inhibition of Hippocampal Progenitor Cell Proliferation Induced by Thiamine Deficiency. *Biomed Res Int*, doi:Artn 572915 10.1155/2014/572915 (2014).

49 Barbagallo, R. P., Oxborough, K., Pallett, K. E. & Baker, N. R. Rapid, noninvasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant physiology* **132**, 485-493, doi:10.1104/pp.102.018093 (2003).

50 von Caemmerer, S. *et al.* Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *J Exp Bot* **55**, 1157-1166, doi:10.1093/jxb/erh128 (2004).

51 Baker, N. R., Oxborough, K., Lawson, T. & Morison, J. I. L. High resolution imaging of photosynthetic activities of tissues, cells and chloroplasts in leaves. *Journal of Experimental Botany* **52**, 615-621 (2001).

52 Oxborough, K. & Baker, N. R. An evaluation of the potential triggers of photoinactivation of photosystem II in the context of a Stern–Volmer model for downregulation and the reversible radical pair equilibrium model. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **355**, 1489-1498 (2000).

53 Lawson, T., Lefebvre, S., Baker, N. R., Morison, J. I. L. & Raines, C. A. Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and CO(2). *J Exp Bot* **59**, 3609-3619, doi:10.1093/jxb/ern211 (2008).

54 Farquhar, G., von Caemmerer, S. v. & Berry, J. A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta* **149**, 78-90 (1980).

55 Sharkey, T. D., Bernacchi, C. J., Farquhar, G. D. & Singsaas, E. L. Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant Cell Environ* **30**, 1035-1040, doi:10.1111/j.1365-3040.2007.01710.x (2007).

56 Vialet-Chabrand, S., Matthews, J. S. A., Simkin, A. J., Raines, C. A. & Lawson, T. Importance of Fluctuations in Light on Plant Photosynthetic Acclimation. *Plant physiology* **173**, 2163-2179, doi:10.1104/pp.16.01767 (2017).

57 von Caemmerer, S. & Farquhar, G. D. Some Relationships between the Biochemistry of Photosynthesis and the Gas-Exchange of Leaves. *Planta* **153**, 376-387, doi:Doi 10.1007/Bf00384257 (1981).

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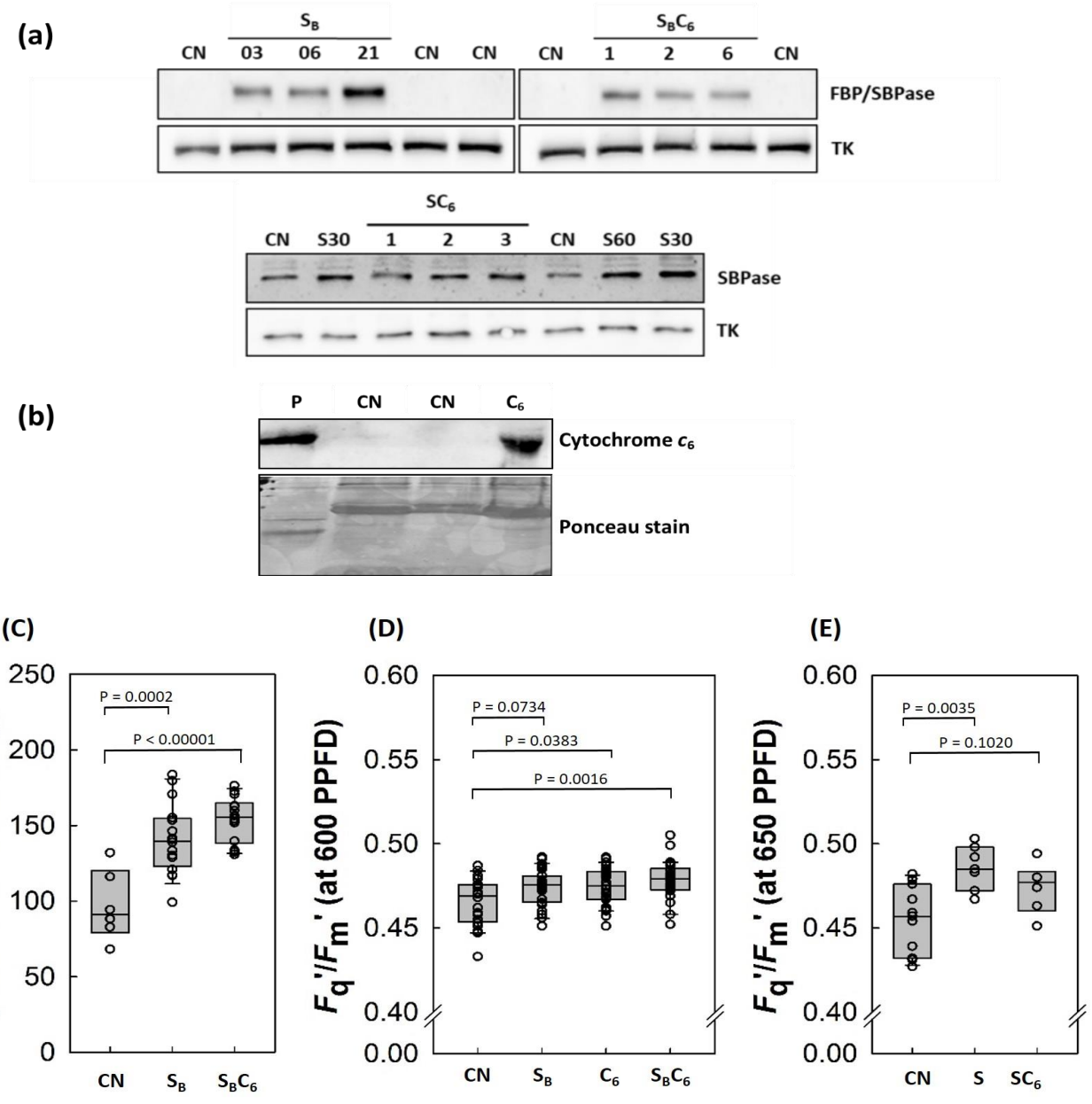
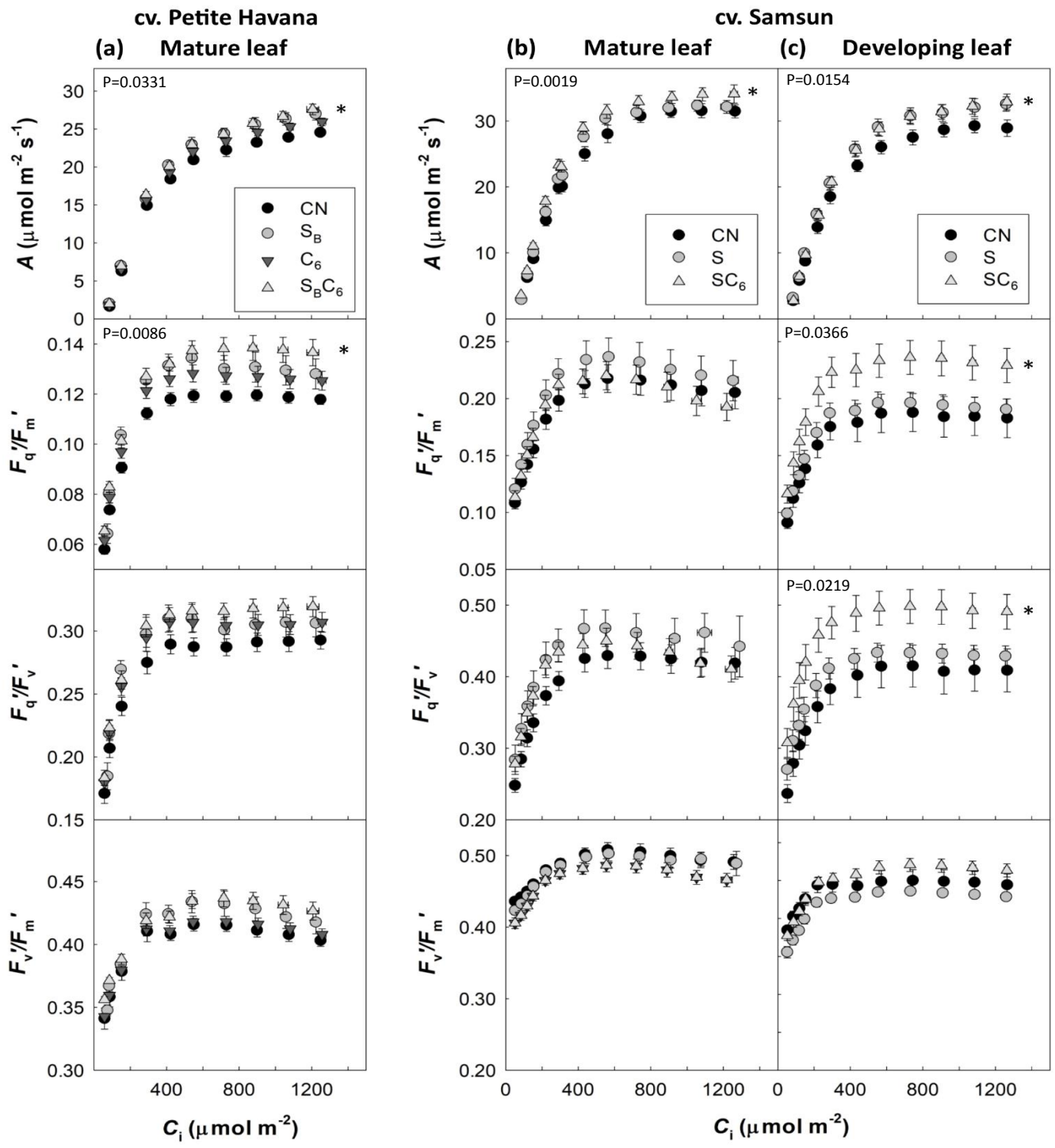
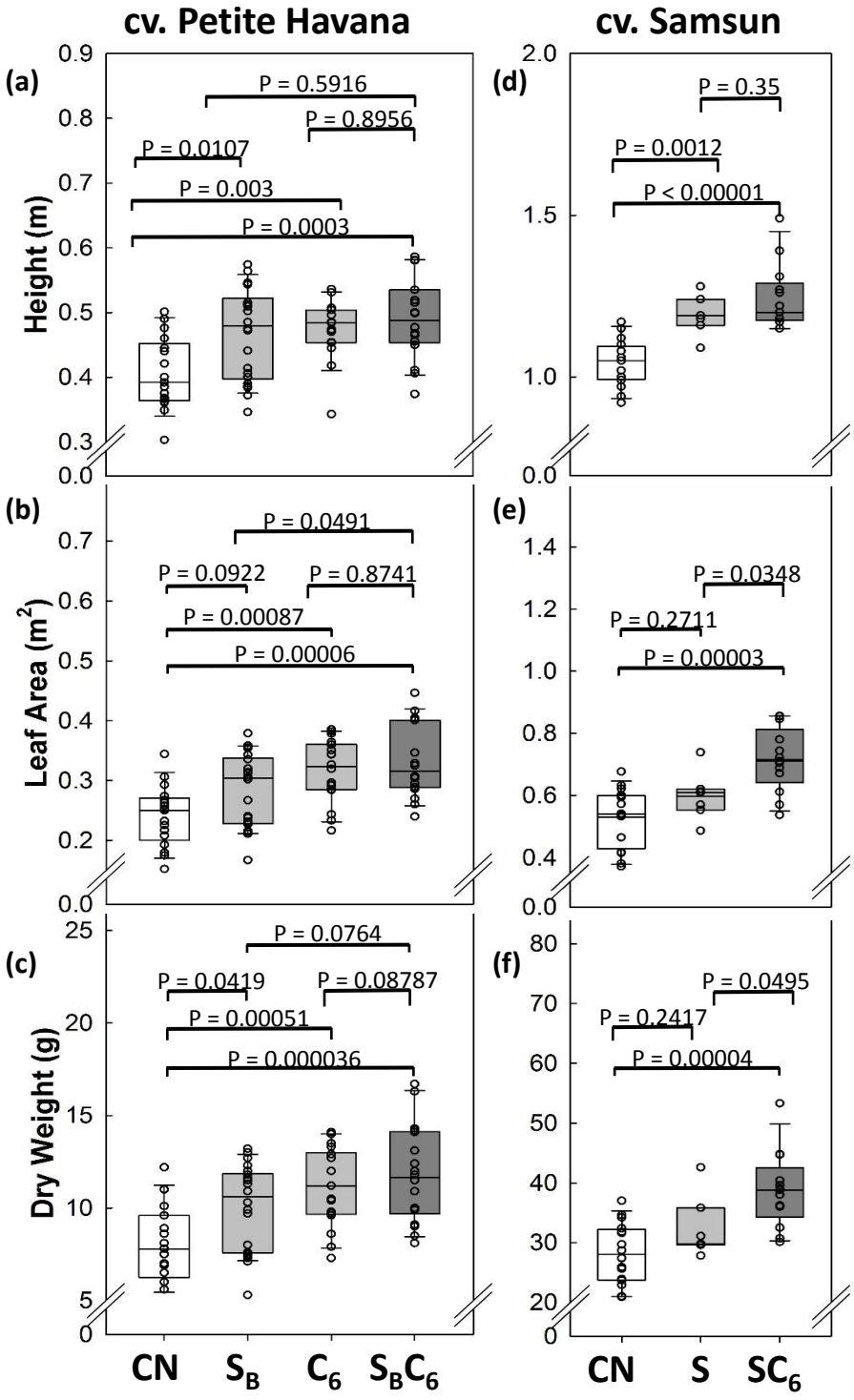
Author contributions

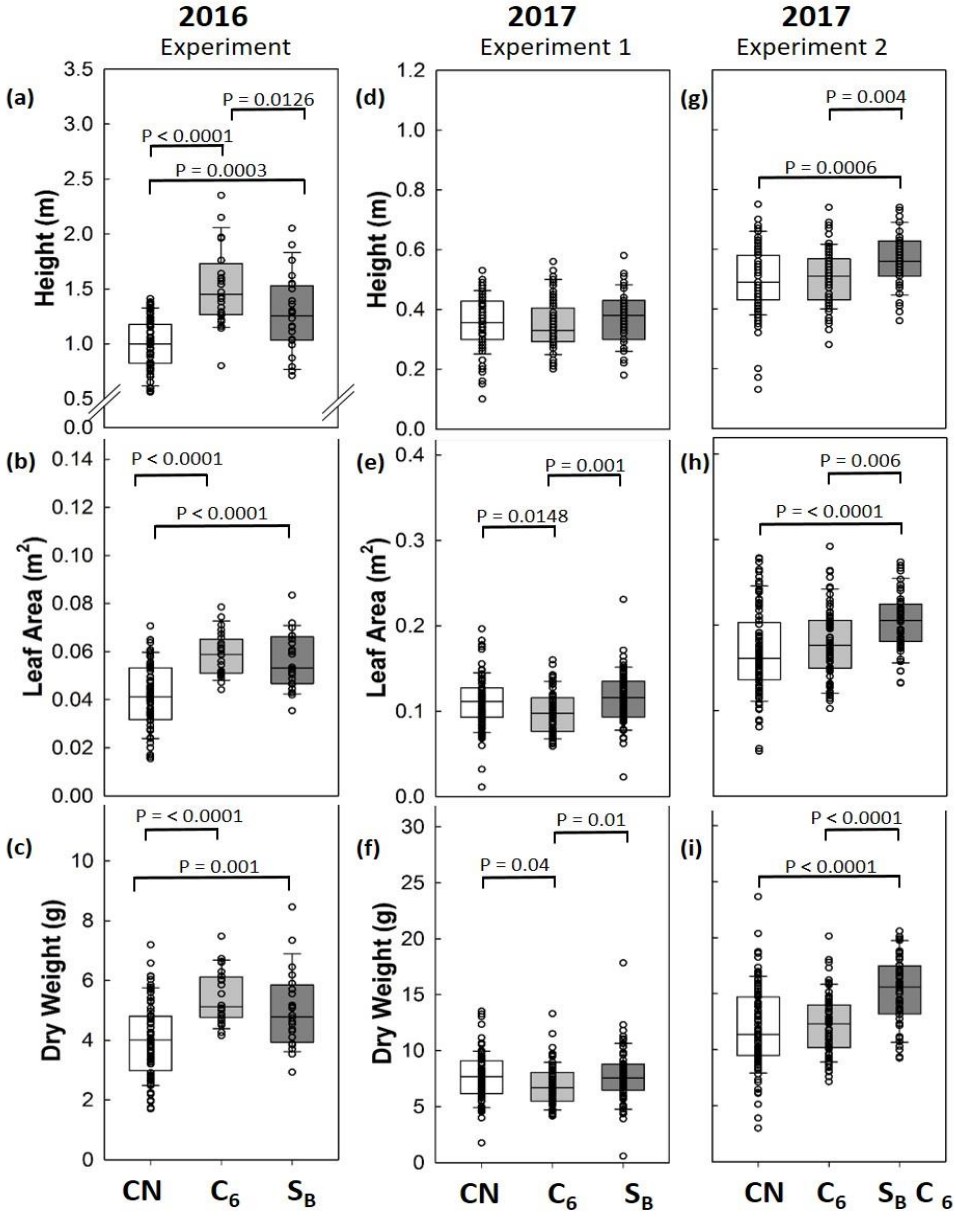
P.E.L.C and A.J.S. generated transgenic plants. P.E.L.C, A.J.S, K.L.B. and S.J.F. performed molecular and biochemical experiments. P.E.L.C, A.J.S and K.L.B carried out plant phenotypic and growth analysis and performed gas exchange measurement, S.V.C. made the measurements of photosynthesis during light induction. A.J.S and S.J.F performed enzyme assays on selected lines; all authors carried out data analysis on their respective contributions; C.A.R and T.L designed and supervised the research; P.E.L.C., A.J.S and C.A.R wrote the manuscript, TL contributed to editing of the manuscript and finalising of figures. P.E.L.C, K.L.B. and A.J.S contributed equally to the completion of this work.

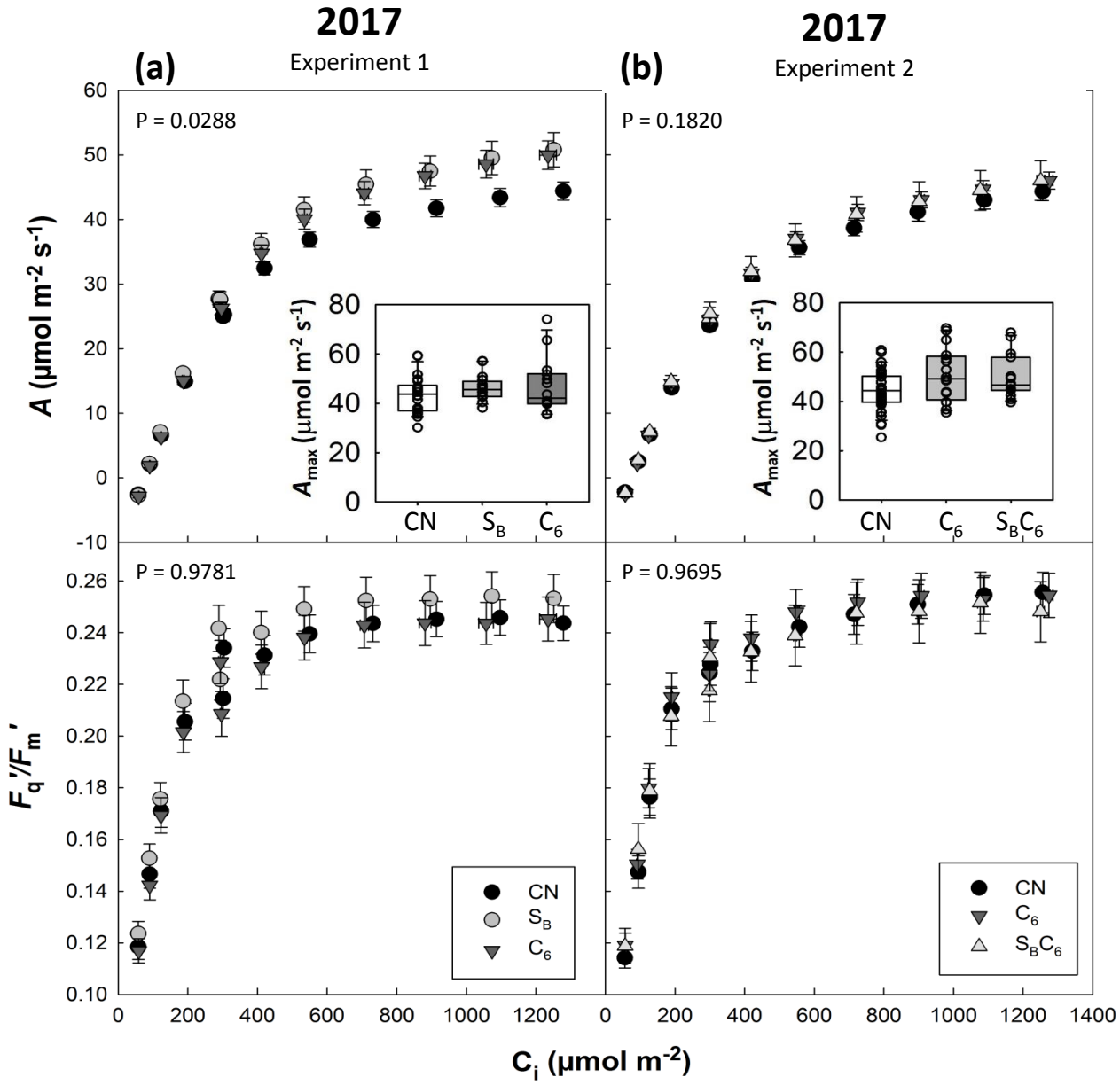
Competing interests: The authors declare no competing financial interests


(a)
S_B
S_BC_6
CN 03 06 21 CN CN CN 1 2 6 CN
FBP/SBPase
TK
SC_6
CN S30 1 2 3 CN S60 S30
SBPase
TK
(b)
P CN CN C_6
Cytochrome c_6
Ponceau stain
(C)
FBPase activity (relative to TK activity)
P = 0.0002
P < 0.00001
CN S_B S_BC_6
(D)
F_q'/F_m' (at 600 PPFD)
P = 0.0734
P = 0.0383
P = 0.0016
CN S_B C_6 S_BC_6
(E)
F_q'/F_m' (at 650 PPFD)
P = 0.0035
P = 0.1020
CN S SC_6


cv. Petite Havana
(a) Mature leaf
cv. Samsun
(b) Mature leaf
(c) Developing leaf
P=0.0331
P=0.0019
P=0.0154
P=0.0086
P=0.0366
P=0.0219
CN
S_B
C_6
S_BC_6
S
SC_6
A (μmol m^-2 s^-1)
F_q'/F_m'
F_q'/F_v'
F_v'/F_m'
C_i (μmol m^-2)


cv. Petite Havana
cv. Samsun
(a)
(b)
(c)
(d)
(e)
(f)
Height (m)
Leaf Area (m²)
Dry Weight (g)
P = 0.5916
P = 0.8956
P = 0.0107
P = 0.003
P = 0.0003
P = 0.35
P = 0.0012
P < 0.00001
P = 0.0491
P = 0.0922
P = 0.8741
P = 0.00087
P = 0.00006
P = 0.0348
P = 0.2711
P = 0.00003
P = 0.0764
P = 0.0419
P = 0.08787
P = 0.00051
P = 0.000036
P = 0.0495
P = 0.2417
P = 0.00004
CN
S_B
C_6
S_BC_6
CN
S
SC_6


2016
Experiment
2017
Experiment 1
2017
Experiment 2
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Height (m)
Leaf Area (m²)
Dry Weight (g)
P = 0.0126
P < 0.0001
P = 0.0003
P = 0.004
P = 0.0006
P < 0.0001
P < 0.0001
P = 0.001
P = 0.0148
P = 0.006
P = < 0.0001
P = < 0.0001
P = 0.001
P = 0.04
P = 0.01
P < 0.0001
P < 0.0001
CN
C6
SB
C 6


2017
(a)
Experiment 1
2017
(b)
Experiment 2
P = 0.0288
P = 0.1820
P = 0.9781
P = 0.9695
A (µmol m-2 s-1)
Fq'/Fm'
Ci (µmol m-2)
Amax (µmol m-2 s-1)
CN
SB
C6
SBC6

2017

Experiment 2